

**METHODS FOR RAPID DETECTION AND IDENTIFICATION OF BACTERIAL  
BIOAGENTS**

**FIELD OF THE INVENTION**

5 The present invention relates to methods for rapid detection and identification of bioagents from environmental, clinical or other samples. The methods provide for detection and characterization of a unique mass or unique base composition signature (BCS) from any bioagent, including bacteria and viruses. The unique mass or unique BCS is used to rapidly identify the bioagent(s).

10

**BACKGROUND OF THE INVENTION**

Rapid and definitive microbial identification is desirable for a variety of industrial, medical, environmental, quality, and research reasons. Traditionally, the microbiology laboratory has functioned to identify the etiologic agents of infectious diseases through direct 15 examination and culture of specimens. Since the mid-1980s, researchers have repeatedly demonstrated the practical utility of molecular biology techniques, many of which form the basis of clinical diagnostic assays. Some of these techniques include nucleic acid hybridization analysis, restriction enzyme analysis, genetic sequence analysis, and separation and purification of nucleic acids (See, e.g., J. Sambrook, E. F. Fritsch, and T. Maniatis, 20 Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989). These procedures, in general, are time-consuming and tedious. Another option is the polymerase chain reaction (PCR) or other amplification procedure that amplifies a specific target DNA sequence based on the flanking primers used. Finally, detection and data analysis convert the hybridization event into an analytical result.

25 Other techniques for detection of bioagents include high-resolution mass spectrometry (MS), low-resolution MS, fluorescence, radioiodination, DNA chips and antibody techniques. None of these techniques is entirely satisfactory.

Mass spectrometry provides detailed information about the molecules being analyzed, including high mass accuracy. It is also a process that can be easily automated. However, 30 high-resolution MS alone fails to perform against unknown or bioengineered agents, or in environments where there is a high background level of bioagents ("cluttered" background). Low-resolution MS can fail to detect some known agents, if their spectral lines are sufficiently weak or sufficiently close to those from other living organisms in the sample.

DNA chips with specific probes can only determine the presence or absence of specifically anticipated organisms. Because there are hundreds of thousands of species of benign bacteria, some very similar in sequence to threat organisms, even arrays with 10,000 probes lack the breadth needed to detect a particular organism.

5       Antibodies face more severe diversity limitations than arrays. If antibodies are designed against highly conserved targets to increase diversity, the false alarm problem will dominate, again because threat organisms are very similar to benign ones. Antibodies are only capable of detecting known agents in relatively uncluttered environments.

Several groups have described detection of PCR products using high resolution  
10      electrospray ionization-Fourier transform-ion cyclotron resonance mass spectrometry (ESI-  
FT-ICR MS). Accurate measurement of exact mass combined with knowledge of the number  
of at least one nucleotide allowed calculation of the total base composition for PCR duplex  
products of approximately 100 base pairs. (Aaserud et al., *J. Am. Soc. Mass Spec.* 7:1266-  
1269, 1996; Muddiman et al., *Anal. Chem.* 69:1543-1549, 1997; Wunschel et al., *Anal.*  
15      *Chem.* 70:1203-1207, 1998; Muddiman et al., *Rev. Anal. Chem.* 17:1-68, 1998). Electrospray  
ionization-Fourier transform-ion cyclotron resistance (ESI-FT-ICR) MS may be used to  
determine the mass of double-stranded, 500 base-pair PCR products via the average  
molecular mass (Hurst et al., *Rapid Commun. Mass Spec.* 10:377-382, 1996). The use of  
20      matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry  
for characterization of PCR products has been described. (Muddiman et al., *Rapid Commun.*  
*Mass Spec.* 13:1201-1204, 1999). However, the degradation of DNAs over about 75  
nucleotides observed with MALDI limited the utility of this method.

U.S. Patent No. 5,849,492 describes a method for retrieval of phylogenetically  
informative DNA sequences that comprise searching for a highly divergent segment of  
25      genomic DNA surrounded by two highly conserved segments, designing the universal  
primers for PCR amplification of the highly divergent region, amplifying the genomic DNA  
by PCR technique using universal primers, and then sequencing the gene to determine the  
identity of the organism.

U.S. Patent No. 5,965,363 discloses methods for screening nucleic acids for  
30      polymorphisms by analyzing amplified target nucleic acids using mass spectrometric  
techniques and to procedures for improving mass resolution and mass accuracy of these  
methods.

WO 99/14375 describes methods, PCR primers and kits for use in analyzing preselected DNA tandem nucleotide repeat alleles by mass spectrometry.

WO 98/12355 discloses methods of determining the mass of a target nucleic acid by mass spectrometric analysis, by cleaving the target nucleic acid to reduce its length, making 5 the target single-stranded and using MS to determine the mass of the single-stranded shortened target. Also disclosed are methods of preparing a double-stranded target nucleic acid for MS analysis comprising amplification of the target nucleic acid, binding one of the strands to a solid support, releasing the second strand and then releasing the first strand, which is then analyzed by MS. Kits for target nucleic acid preparation are also provided.

10 PCT WO97/33000 discloses methods for detecting mutations in a target nucleic acid by nonrandomly fragmenting the target into a set of single-stranded nonrandom length fragments and determining their masses by MS.

15 U.S. Patent No. 5,605,798 describes a fast and highly accurate mass spectrometer-based process for detecting the presence of a particular nucleic acid in a biological sample for diagnostic purposes.

20 WO 98/21066 describes processes for determining the sequence of a particular target nucleic acid by mass spectrometry. Processes for detecting a target nucleic acid present in a biological sample by PCR amplification and mass spectrometry detection are disclosed, as are methods for detecting a target nucleic acid in a sample by amplifying the target with primers that contain restriction sites and tags, extending and cleaving the amplified nucleic acid, and detecting the presence of extended product, wherein the presence of a DNA fragment of a mass different from wild-type is indicative of a mutation. Methods of sequencing a nucleic acid via mass spectrometry methods are also described.

25 WO 97/37041, WO 99/31278 and US Patent No. 5,547,835 describe methods of sequencing nucleic acids using mass spectrometry. US Patent Nos. 5,622,824, 5,872,003 and 5,691,141 describe methods, systems and kits for exonuclease-mediated mass spectrometric sequencing.

30 Thus, there is a need for a method for bioagent detection and identification which is both specific and rapid, and in which no nucleic acid sequencing is required. The present invention addresses this need.

## SUMMARY OF THE INVENTION

One embodiment of the present invention is a method of identifying an unknown bioagent in a container comprising a) contacting nucleic acid from the bioagent with at least one pair of oligonucleotide primers that hybridize to sequences of the nucleic acid and flank a variable nucleic acid sequence; b) amplifying the variable nucleic acid sequence to produce an amplification product; c) determining the molecular mass of the amplification product; and d) comparing the molecular mass to one or more molecular masses of amplification products obtained by performing steps a)-c) on a plurality of known organisms, wherein a match identifies the unknown bioagent in the container. In some embodiments, the container is a box or envelope. In one aspect of the invention, the sequences to which the at least one pair of oligonucleotide primers hybridize are highly conserved. Preferably, the amplifying step comprises polymerase chain reaction. Alternatively, the amplifying step comprises ligase chain reaction or strand displacement amplification. In one aspect of the invention, the bioagent is a bacterium, virus, parasite, fungi, cell or spore. Advantageously, the nucleic acid is ribosomal RNA. In another aspect, the nucleic acid encodes RNase P or an RNA-dependent RNA polymerase. Preferably, the amplification product is ionized prior to molecular mass determination. The method may further comprise the step of isolating nucleic acid from the bioagent prior to contacting the nucleic acid with the at least one pair of oligonucleotide primers. The method may further comprise the step of performing steps a)-d) using a different oligonucleotide primer pair and comparing the results to one or more molecular mass amplification products obtained by performing steps a)-c) on a different plurality of known organisms from those in step d). Preferably, the one or more molecular mass is contained in a database of molecular masses. In another aspect of the invention, the amplification product is ionized by electrospray ionization, matrix-assisted laser desorption or fast atom bombardment. Advantageously, the molecular mass is determined by mass spectrometry. Preferably, the mass spectrometry is Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), ion trap, quadrupole, magnetic sector, time of flight (TOF), Q-TOF or triple quadrupole. The method may further comprise performing step b) in the presence of an analog of adenine, thymidine, guanosine or cytidine having a different molecular weight than adenosine, thymidine, guanosine or cytidine. In one aspect, the oligonucleotide primer comprises a base analog or substitute base at positions 1 and 2 of each triplet within the primer, wherein the base analog or substitute base binds with increased affinity to its complement compared to the native base. Preferably, the primer comprises a

universal base at position 3 of each triplet within the primer. The base analog or substitute base may be 2,6-diaminopurine, propyne T, propyne G, phenoxyazines or G-clamp. Preferably, the universal base is inosine, guanidine, uridine, 5-nitroindole, 3-nitropyrrole, dP or dK, or 1-(2-deoxy- $\beta$ -D-ribofuranosyl)-imidazole-4-carboxamide.

5 Another embodiment of the present invention is a method of identifying an unknown bioagent in a container comprising a) contacting nucleic acid from the bioagent with at least one pair of oligonucleotide primers that hybridize to sequences of the nucleic acid and flank a variable nucleic acid sequence; b) amplifying the variable nucleic acid sequence to produce an amplification product; c) determining the base composition of the amplification product; 10 and d) comparing the base composition to one or more base compositions of amplification products obtained by performing steps a)-c) on a plurality of known organisms, wherein a match identifies the unknown bioagent in the container. In some embodiments, the container is a box or envelope. In one aspect of the invention, the sequences to which the at least one pair of oligonucleotide primers hybridize are highly conserved. Preferably, the amplifying 15 step comprises polymerase chain reaction. Alternatively, the amplifying step comprises ligase chain reaction or strand displacement amplification. In one aspect of the invention, the bioagent is a bacterium, virus, parasite, fungi, cell or spore. Advantageously, the nucleic acid is ribosomal RNA. In another aspect, the nucleic acid encodes RNase P or an RNA-dependent RNA polymerase. Preferably, the amplification product is ionized prior to 20 molecular mass determination. The method may further comprise the step of isolating nucleic acid from the bioagent prior to contacting the nucleic acid with the at least one pair of oligonucleotide primers. The method may further comprise the step of performing steps a)-d) using a different oligonucleotide primer pair and comparing the results to one or more base composition signatures of amplification products obtained by performing steps a)-c) on a 25 different plurality of known organisms from those in step d). Preferably, the one or more base compositions is contained in a database of base compositions. In another aspect of this preferred embodiment, the amplification product is ionized by electrospray ionization, matrix-assisted laser desorption or fast atom bombardment. Advantageously, the molecular mass is determined by mass spectrometry. Preferably, the mass spectrometry is Fourier 30 transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), ion trap, quadrupole, magnetic sector, time of flight (TOF), Q-TOF or triple quadrupole. The method may further comprise performing step b) in the presence of an analog of adenine, thymidine, guanosine or cytidine having a different molecular weight than adenosine, thymidine, guanosine or

cytidine. In one aspect, the oligonucleotide primer comprises a base analog or substitute base at positions 1 and 2 of each triplet within the primer, wherein the base analog or substitute base binds with increased affinity to its complement compared to the native base. Preferably, the primer comprises a universal base at position 3 of each triplet within the primer. The base 5 analog or substitute base may be 2,6-diaminopurine, propyne T, propyne G, phenoxazines or G-clamp. Preferably, the universal base is inosine, guanidine, uridine, 5-nitroindole, 3-nitropyrrole, dP or dK, or 1-(2-deoxy- $\beta$ -D-ribofuranosyl)-imidazole-4-carboxamide.

#### BRIEF DESCRIPTION OF THE DRAWINGS

10 Figures 1A-1H and Figure 2 are consensus diagrams that show examples of conserved regions from 16S rRNA (Fig. 1A, 1A-2, 1A-3, 1A-4, 1B, 1B-1, and 1B-2), 23S rRNA (3'-half, Fig. 1C-1, 1C-2, and 1D; 5'-half, Fig. 1E-F), 23S rRNA Domain I (Fig. 1G), 23S rRNA Domain IV (Fig. 1H) and 16S rRNA Domain III (Fig. 2) which are suitable for use in the present invention. Where there is overlap or redundancy between the figures, the overlap is 15 simply provided as an orientation aid and no additional members of the sequence are implied thereby. Lines with arrows are examples of regions to which intelligent primer pairs for PCR are designed. The label for each primer pair represents the starting and ending base number of the amplified region on the consensus diagram. Bases in capital letters are greater than 95% conserved; bases in lower case letters are 90-95% conserved, filled circles are 80-90% 20 conserved; and open circles are less than 80% conserved. The label for each primer pair represents the starting and ending base number of the amplified region on the consensus diagram. The nucleotide sequence of the 16S rRNA consensus sequence is SEQ ID NO:3 and the nucleotide sequence of the 23S rRNA consensus sequence is SEQ ID NO:4.

25 Figure 2 shows a typical primer amplified region from the 16S rRNA Domain III shown in Figure 1C.

Figure 3 is a schematic diagram showing conserved regions in RNase P. Bases in capital letters are greater than 90% conserved; bases in lower case letters are 80-90% conserved; filled circles designate bases which are 70-80% conserved; and open circles designate bases that are less than 70% conserved.

30 Figure 4 is a schematic diagram of base composition signature determination using nucleotide analog “tags” to determine base composition signatures.

Figure 5 shows the deconvoluted mass spectra of a *Bacillus anthracis* region with and without the mass tag phosphorothioate A (A\*). The two spectra differ in that the measured

molecular weight of the mass tag-containing sequence is greater than the unmodified sequence.

Figure 6 shows base composition signature (BCS) spectra from PCR products from *Staphylococcus aureus* (S. aureus 16S\_1337F) and *Bacillus anthracis* (B. anthr. 5 16S\_1337F), amplified using the same primers. The two strands differ by only two (AT→CG) substitutions and are clearly distinguished on the basis of their BCS.

Figure 7 shows that a single difference between two sequences (A<sub>14</sub> in *B. anthracis* vs. A<sub>15</sub> in *B. cereus*) can be easily detected using ESI-TOF mass spectrometry.

Figure 8 is an ESI-TOF of *Bacillus anthracis* spore coat protein sspE 56mer plus 10 calibrant. The signals unambiguously identify *B. anthracis* versus other *Bacillus* species.

Figure 9 is an ESI-TOF of a *B. anthracis* synthetic 16S\_1228 duplex (reverse and forward strands). The technique easily distinguishes between the forward and reverse strands.

Figure 10 is an ESI-FTICR-MS of a synthetic *B. anthracis* 16S\_1337 46 base pair 15 duplex.

Figure 11 is an ESI-TOF-MS of a 56mer oligonucleotide (3 scans) from the *B. anthracis* saspB gene with an internal mass standard. The internal mass standards are designated by asterisks.

Figure 12 is an ESI-TOF-MS of an internal standard with 5 mM TBA-TFA buffer 20 showing that charge stripping with tributylammonium trifluoroacetate reduces the most abundant charge state from [M-8H<sup>+</sup>]<sup>8-</sup> to [M-3H<sup>+</sup>]<sup>3-</sup>.

## DETAILED DESCRIPTION OF EMBODIMENTS

The present invention provides a combination of a non-PCR biomass detection mode, 25 preferably high-resolution MS, with PCR-based BCS or mass technology using “intelligent primers” which hybridize to conserved sequence regions of nucleic acids derived from a bioagent and which bracket variable sequence regions that uniquely identify the bioagent. The high-resolution MS technique is used to determine the molecular mass and/or base 30 composition signature (BCS) of the amplified sequence region. This unique mass or unique “base composition signature” (BCS) is then input to a maximum-likelihood detection algorithm for matching against a database of masses or base composition signatures in the same amplified region. The present method combines PCR-based amplification technology (which provides specificity) and a molecular mass detection mode (which provides speed and

does not require nucleic acid sequencing of the amplified target sequence) for bioagent detection and identification.

The present method allows extremely rapid and accurate detection and identification of bioagents compared to existing methods. Furthermore, this rapid detection and 5 identification is possible even when sample material is impure. The method leverages ongoing biomedical research in virulence, pathogenicity, drug resistance and genome sequencing into a method which provides greatly improved sensitivity, specificity and reliability compared to existing methods, with lower rates of false positives. Thus, the methods are useful in a wide variety of fields, including, but not limited to, those fields 10 discussed below.

In some embodiments of the invention, the methods disclosed herein can be used for environmental testing. Detection and discrimination of pathogenic vs. non-pathogenic bacteria, viruses, parasites, fungi and the like, in samples of water, land, air, or other samples, can be carried out. Water samples can be obtained from, for example, lakes, rivers, oceans, 15 streams, water treatment systems, rainwater, groundwater, water table, reservoirs, wells, bottled water, and the like. Air samples can be obtained from ventilation systems, airplane cabins, schools, hospitals, mass transit locations such as subways, train stations, airports, and the like. Conditions such as sick building syndrome can be detected. Land samples can be obtained from any location.

20 In other embodiments of the invention, the methods disclosed herein can be used to screen blood and other bodily fluids and tissues for pathogenic and non-pathogenic bacteria, viruses, parasites, fungi and the like. Animal samples, including but not limited to, blood and other bodily fluid and tissue samples, can be obtained from living animals, who are either known or not known to or suspected of having a disease, infection, or condition. Alternately, 25 animal samples such as blood and other bodily fluid and tissue samples can be obtained from deceased animals. Blood samples can be further separated into plasma or cellular fractions and further screened as desired. Bodily fluids and tissues can be obtained from any part of the animal or human body. Animal samples can be obtained from, for example, mammals and humans.

30 In other embodiments of the invention, the methods disclosed herein can be used for forensics. For example, medical examiners can use the present invention to determine the cause of death. In addition, epidemiologists, for example, can use the present methods to determine the geographic origin of a particular strain of bacteria or virus. For example, a

particular strain of bacteria or virus may have a sequence difference that is associated with a particular area of a country or the world and identification of such a sequence difference can lead to the identification of the geographic origin and epidemiological tracking of the spread of the particular disease, disorder or condition associated with the detected virus or bacteria.

5 In addition, carriers of particular DNA or diseases, such as mammals, non-mammals, birds, insects, and plants, can be tracked by screening SNPs, VNTRs, or polyA, for example. Diseases, such as malaria, can be tracked by screening commensals, such as mosquitos.

In other embodiments of the invention, the methods disclosed herein can be used for detecting the presence of pathogenic and non-pathogenic bacteria, viruses, parasites, fungi

10 and the like in samples of foodstuff or cosmetics. For example, food and wine can be examined for the presence of pathogenic and non-pathogenic bacteria, viruses, parasites, fungi and the like. Particular types of foods susceptible to bioagent contamination, such as agricultural products, meat products and eggs, can be examined for pathogenic organisms such as *E. coli* and *Salmonella* species. Such examination procedures can be used by, for 15 example, the wholesalers of foodstuffs and beverages, or by regulatory agencies such as the U.S. Department of Agriculture and the Food and Drug Administration. In addition, grapes and wines, for example, can be examined using the present methods to detect particular strains of bacteria or yeast that may indicate a particular time upon which to harvest the grapes or alter the wine-making process.

20 In other embodiments of the invention, the methods disclosed herein can be used for detecting the presence of bioagents in a container, such as a package, box, envelope, mail tube, railroad box car, and the like. For example, mail and package delivery entities and agencies, both domestic and abroad, as well investigative agencies such as the FBI and ATF can use the present methods to detect bioagents in containers.

25 In other embodiments of the invention, the methods disclosed herein can be used for detecting the presence of pathogenic and non-pathogenic bacteria, viruses, parasites, fungi and the like in organ donors and/or in organs from donors. Such examination can result in the prevention of the transfer of, for example, viruses such as West Nile virus, hepatitis viruses, human immunodeficiency virus, and the like from a donor to a recipient via a transplanted 30 organ. The methods disclosed herein can also be used for detection of host versus graft or graft versus host rejection issues related to organ donors by detecting the presence of particular antigens in either the graft or host known or suspected of causing such rejection. In

particular, the bioagents in this regard are the antigens of the major histocompatibility complex, such as the HLA antigens.

In other embodiments of the invention, the methods disclosed herein can be used for detection and identification of livestock infections such as, for example, mad cow disease, 5 hoof and mouth disease, and the like. Livestock includes, but is not limited to, cows, pigs, sheep, chickens, turkeys, goats, and other farm animals.

In other embodiments of the invention, the methods disclosed herein can be used for pharmacogenetic analysis and medical diagnosis including, but not limited to, cancer diagnosis based on mutations and polymorphisms, drug resistance and susceptibility testing, 10 screening for and/or diagnosis of genetic diseases and conditions, and diagnosis of infectious diseases and conditions. The present methods can also be used to detect and track emerging infectious diseases, such as West Nile virus infection, mad cow disease, and HIV-related diseases.

The present methods can be used to detect and classify any biological agent, including 15 bacteria, viruses, fungi and toxins. As one example, where the agent is a biological threat, the information obtained is used to determine practical information needed for countermeasures, including toxin genes, pathogenicity islands and antibiotic resistance genes. In addition, the methods can be used to identify natural or deliberate engineering events including chromosome fragment swapping, molecular breeding (gene shuffling) and emerging 20 infectious diseases.

Bacteria have a common set of absolutely required genes. About 250 genes are present in all bacterial species (*Proc. Natl. Acad. Sci. U.S.A.* **93**:10268, 1996; *Science* **270**:397, 1995), including tiny genomes like *Mycoplasma*, *Ureaplasma* and *Rickettsia*. These genes encode proteins involved in translation, replication, recombination and repair, 25 transcription, nucleotide metabolism, amino acid metabolism, lipid metabolism, energy generation, uptake, secretion and the like. Examples of these proteins are DNA polymerase III beta, elongation factor TU, heat shock protein groEL, RNA polymerase beta, phosphoglycerate kinase, NADH dehydrogenase, DNA ligase, DNA topoisomerase and elongation factor G. Operons can also be targeted using the present method. One example of 30 an operon is the bfp operon from enteropathogenic *E. coli*. Multiple core chromosomal genes can be used to classify bacteria at a genus or genus species level to determine if an organism has threat potential. The method can also be used to detect pathogenicity markers (plasmid or

chromosomal) and antibiotic resistance genes to confirm the threat potential of an organism and to direct countermeasures.

A theoretically ideal bioagent detector would identify, quantify, and report the complete nucleic acid sequence of every bioagent that reached the sensor. The complete 5 sequence of the nucleic acid component of a pathogen would provide all relevant information about the threat, including its identity and the presence of drug-resistance or pathogenicity markers. This ideal has not yet been achieved. However, the present invention provides a straightforward strategy for obtaining information with the same practical value using base composition signatures (BCS). While the base composition of a gene fragment is not as 10 information-rich as the sequence itself, there is no need to analyze the complete sequence of the gene if the short analyte sequence fragment is properly chosen. A database of reference sequences can be prepared in which each sequence is indexed to a unique base composition signature, so that the presence of the sequence can be inferred with accuracy from the presence of the signature. The advantage of base composition signatures is that they can be 15 quantitatively measured in a massively parallel fashion using multiplex PCR (PCR in which two or more primer pairs amplify target sequences simultaneously) and mass spectrometry. These multiple primer amplified regions uniquely identify most threat and ubiquitous background bacteria and viruses. In addition, cluster-specific primer pairs distinguish important local clusters (e.g., anthracis group).

20 In the context of this invention, a “bioagent” is any organism, living or dead, or a nucleic acid derived from such an organism. Examples of bioagents include but are not limited to cells (including but not limited to human clinical samples, bacterial cells and other pathogens) viruses, parasites, fungi, toxin genes and bioregulating compounds. Samples may be alive or dead or in a vegetative state (for example, vegetative bacteria or spores) and may 25 be encapsulated or bioengineered.

As used herein, a “base composition signature” (BCS) is the exact base composition from selected fragments of nucleic acid sequences that uniquely identifies the target gene and source organism. BCS can be thought of as unique indexes of specific genes.

As used herein, “intelligent primers” are primers which bind to sequence regions 30 which flank an intervening variable region. In a preferred embodiment, these sequence regions which flank the variable region are highly conserved among different species of bioagent. For example, the sequence regions may be highly conserved among all *Bacillus* species. By the term “highly conserved”, it is meant that the sequence regions exhibit

between about 80-100%, more preferably between about 90-100% and most preferably between about 95-100% identity. Examples of intelligent primers which amplify regions of the 16S and 23S rRNA are shown in Figures 1A-1I. A typical primer amplified region in 16S rRNA is shown in Figure 2. The arrows represent primers which bind to highly conserved regions which flank a variable region in 16S rRNA domain III. The amplified region is the stem-loop structure under "1100-1188."

One main advantage of the detection methods of the present invention is that the primers need not be specific for a particular bacterial species, or even genus, such as *Bacillus* or *Streptomyces*. Instead, the primers recognize highly conserved regions across hundreds of bacterial species including, but not limited to, the species described herein. Thus, the same primer pair can be used to identify any desired bacterium because it will bind to the conserved regions which flank a variable region specific to a single species, or common to several bacterial species, allowing nucleic acid amplification of the intervening sequence and determination of its molecular weight and base composition. For example, the 16S\_971-1062, 16S\_1228-1310 and 16S\_1100-1188 regions are 98-99% conserved in about 900 species of bacteria (16S=16S rRNA, numbers indicate nucleotide position). In one embodiment of the present invention, primers used in the present method bind to one or more of these regions or portions thereof.

The present invention provides a combination of a non-PCR biomass detection mode, preferably high-resolution MS, with nucleic acid amplification-based BCS technology using "intelligent primers" which hybridize to conserved regions and which bracket variable regions that uniquely identify the bioagent(s). Although the use of PCR is preferred, other nucleic acid amplification techniques may also be used, including ligase chain reaction (LCR) and strand displacement amplification (SDA). The high-resolution MS technique allows separation of bioagent spectral lines from background spectral lines in highly cluttered environments. The resolved spectral lines are then translated to BCS which are input to a maximum-likelihood detection algorithm matched against spectra for one or more known BCS. Preferably, the bioagent BCS spectrum is matched against one or more databases of BCS from vast numbers of bioagents. Preferably, the matching is done using a maximum-likelihood detection algorithm.

In a preferred embodiment, base composition signatures are quantitatively measured in a massively parallel fashion using the polymerase chain reaction (PCR), preferably multiplex PCR, and mass spectrometric (MS) methods. Sufficient quantities of nucleic acids

must be present for detection of bioagents by MS. A wide variety of techniques for preparing large amounts of purified nucleic acids or fragments thereof are well known to those of skill in the art. PCR requires one or more pairs of oligonucleotide primers which bind to regions which flank the target sequence(s) to be amplified. These primers prime synthesis of a 5 different strand of DNA, with synthesis occurring in the direction of one primer towards the other primer. The primers, DNA to be amplified, a thermostable DNA polymerase (e.g. *Taq* polymerase), the four deoxynucleotide triphosphates, and a buffer are combined to initiate DNA synthesis. The solution is denatured by heating, then cooled to allow annealing of newly added primer, followed by another round of DNA synthesis. This process is typically 10 repeated for about 30 cycles, resulting in amplification of the target sequence.

The “intelligent primers” define the target sequence region to be amplified and analyzed. In one embodiment, the target sequence is a ribosomal RNA (rRNA) gene sequence. With the complete sequences of many of the smallest microbial genomes now available, it is possible to identify a set of genes that defines “minimal life” and identify 15 composition signatures that uniquely identify each gene and organism. Genes that encode core life functions such as DNA replication, transcription, ribosome structure, translation, and transport are distributed broadly in the bacterial genome and are preferred regions for BCS analysis. Ribosomal RNA (rRNA) genes comprise regions that provide useful base composition signatures. Like many genes involved in core life functions, rRNA genes contain 20 sequences that are extraordinarily conserved across bacterial domains interspersed with regions of high variability that are more specific to each species. The variable regions can be utilized to build a database of base composition signatures. The strategy involves creating a structure-based alignment of sequences of the small (16S) and the large (23S) subunits of the rRNA genes. For example, there are currently over 13,000 sequences in the ribosomal RNA 25 database that has been created and maintained by Robin Gutell, University of Texas at Austin, and is publicly available on the Institute for Cellular and Molecular Biology web page ([www.rna.icmb.utexas.edu/](http://www.rna.icmb.utexas.edu/)). There is also a publicly available rRNA database created and maintained by the University of Antwerp, Belgium at [www.rrna.uia.ac.be](http://www.rrna.uia.ac.be).

These databases have been analyzed to determine regions that are useful as base 30 composition signatures. The characteristics of such regions are: a) between about 80 and 100%, preferably > about 95% identity among species of the particular bioagent of interest, of upstream and downstream nucleotide sequences which serve as sequence amplification primer sites; b) an intervening variable region which exhibits no greater than about 5%

identity among species; and c) a separation of between about 30 and 1000 nucleotides, preferably no more than about 50-250 nucleotides, and more preferably no more than about 60-100 nucleotides, between the conserved regions.

Due to their overall conservation, the flanking rRNA primer sequences serve as good 5 “universal” primer binding sites to amplify the region of interest for most, if not all, bacterial species. The intervening region between the sets of primers varies in length and/or composition, and thus provides a unique base composition signature.

It is advantageous to design the “intelligent primers” to be as universal as possible to minimize the number of primers which need to be synthesized, and to allow detection of 10 multiple species using a single pair of primers. These primer pairs can be used to amplify variable regions in these species. Because any variation (due to codon wobble in the 3<sup>rd</sup> position) in these conserved regions among species is likely to occur in the third position of a DNA triplet, oligonucleotide primers can be designed such that the nucleotide corresponding to this position is a base which can bind to more than one nucleotide, referred to herein as a 15 “universal base”. For example, under this “wobble” pairing, inosine (I) binds to U, C or A; guanine (G) binds to U or C, and uridine (U) binds to U or C. Other examples of universal bases include nitroindoles such as 5-nitroindole or 3-nitropyrrole (Loakes et al., *Nucleosides and Nucleotides* 14:1001-1003, 1995), the degenerate nucleotides dP or dK (Hill et al.), an acyclic nucleoside analog containing 5-nitroindazole (Van Aerschot et al., *Nucleosides and 20 Nucleotides* 14:1053-1056, 1995) or the purine analog 1-(2-deoxy-β-D-ribofuranosyl)-imidazole-4-carboxamide (Sala et al., *Nucl. Acids Res.* 24:3302-3306, 1996).

In another embodiment of the invention, to compensate for the somewhat weaker binding by the “wobble” base, the oligonucleotide primers are designed such that the first and second positions of each triplet are occupied by nucleotide analogs which bind with greater 25 affinity than the unmodified nucleotide. Examples of these analogs are 2,6-diaminopurine which binds to thymine, propyne T which binds to adenine and propyne C and phenoxazines, including G-clamp, which binds to G. Propynes are described in U.S. Patent Nos. 5,645,985, 5,830,653 and 5,484,908, the entire contents of which are incorporated herein by reference. Phenoxazines are described in U.S. Patent Nos. 5,502,177, 5,763,588, and 6,005,096, the 30 entire contents of which are incorporated herein by reference. G-clamps are described in U.S. Patent Nos. 6,007,992 and 6,028,183, the entire contents of which are incorporated herein by reference.

In some embodiments of the invention, the methods described herein can be used for defense of germ warfare (allowing immediate identification of the bioagent and appropriate treatment). Bacterial biological warfare agents capable of being detected by the present methods include *Bacillus anthracis* (anthrax), *Yersinia pestis* (pneumonic plague),  
5 *Franciscella tularensis* (tularemia), *Brucella suis*, *Brucella abortus*, *Brucella melitensis* (undulant fever), *Burkholderia mallei* (glanders), *Burkholderia pseudomallei* (melioidosis), *Salmonella typhi* (typhoid fever), *Rickettsia typhi* (epidemic typhus), *Rickettsia prowasekii* (endemic typhus) and *Coxiella burnetii* (Q fever), *Rhodobacter capsulatus*, *Chlamydia pneumoniae*, *Escherichia coli*, *Shigella dysenteriae*, *Shigella flexneri*, *Bacillus cereus*,  
10 *Clostridium botulinum*, *Coxiella burnetii*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, and *Vibrio cholerae*.

Besides 16S and 23S rRNA, other target regions suitable for use in the present invention for detection of bacteria include 5S rRNA and RNase P (Figure 3).

15 Biological warfare fungus biowarfare agents include *coccidioides immitis* (Coccidioidomycosis).

Biological warfare toxin genes capable of being detected by the methods of the present invention include botulism, T-2 mycotoxins, ricin, staph enterotoxin B, shigatoxin, abrin, aflatoxin, *Clostridium perfringens* epsilon toxin, conotoxins, diacetoxyscirpenol, tetrodotoxin and saxitoxin.

20 Biological warfare viral threat agents are mostly RNA viruses (positive-strand and negative-strand), with the exception of smallpox. Every RNA virus is a family of related viruses (quasispecies). These viruses mutate rapidly and the potential for engineered strains (natural or deliberate) is very high. RNA viruses cluster into families that have conserved RNA structural domains on the viral genome (e.g., virion components, accessory proteins) and conserved housekeeping genes that encode core viral proteins including, for single strand positive strand RNA viruses, RNA-dependent RNA polymerase, double stranded RNA helicase, chymotrypsin-like and papain-like proteases and methyltransferases.  
25

30 Examples of (-)-strand RNA viruses include arenaviruses (e.g., sabia virus, lassa fever, Machupo, Argentine hemorrhagic fever, flexal virus), bunyaviruses (e.g., hantavirus, nairovirus, phlebovirus, hantaan virus, Congo-crimean hemorrhagic fever, rift valley fever), and mononegavirales (e.g., filovirus, paramyxovirus, ebola virus, Marburg, equine morbillivirus).

Examples of (+)-strand RNA viruses include picornaviruses (e.g., coxsackievirus, echovirus, human coxsackievirus A, human echovirus, human enterovirus, human poliovirus, hepatitis A virus, human parechovirus, human rhinovirus), astroviruses (e.g., human astrovirus), calciviruses (e.g., chiba virus, chitta virus, human calcivirus, norwalk virus), 5 nidovirales (e.g., human coronavirus, human torovirus), flaviviruses (e.g., dengue virus 1-4, Japanese encephalitis virus, Kyanasur forest disease virus, Murray Valley encephalitis virus, Rocio virus, St. Louis encephalitis virus, West Nile virus, yellow fever virus, hepatitis c virus) and togaviruses (e.g., Chikugunya virus, Eastern equine encephalitis virus, Mayaro virus, O'nyong-nyong virus, Ross River virus, Venezuelan equine encephalitis virus, Rubella 10 virus, hepatitis E virus). The hepatitis C virus has a 5'-untranslated region of 340 nucleotides, an open reading frame encoding 9 proteins having 3010 amino acids and a 3'-untranslated region of 240 nucleotides. The 5'-UTR and 3'-UTR are 99% conserved in hepatitis C viruses.

In one embodiment, the target gene is an RNA-dependent RNA polymerase or a 15 helicase encoded by (+)-strand RNA viruses, or RNA polymerase from a (-)-strand RNA virus. (+)-strand RNA viruses are double stranded RNA and replicate by RNA-directed RNA synthesis using RNA-dependent RNA polymerase and the positive strand as a template. Helicase unwinds the RNA duplex to allow replication of the single stranded RNA. These viruses include viruses from the family picornaviridae (e.g., poliovirus, coxsackievirus, 20 echovirus), togaviridae (e.g., alphavirus, flavivirus, rubivirus), arenaviridae (e.g., lymphocytic choriomeningitis virus, lassa fever virus), coronaviridae (e.g., human respiratory virus) and Hepatitis A virus. The genes encoding these proteins comprise variable and highly conserved regions which flank the variable regions.

In a preferred embodiment, the detection scheme for the PCR products generated 25 from the bioagent(s) incorporates three features. First, the technique simultaneously detects and differentiates multiple (generally about 6-10) PCR products. Second, the technique provides a BCS that uniquely identifies the bioagent from the possible primer sites. Finally, the detection technique is rapid, allowing multiple PCR reactions to be run in parallel.

In one embodiment, the method can be used to detect the presence of antibiotic 30 resistance and/or toxin genes in a bacterial species. For example, *Bacillus anthracis* comprising a tetracycline resistance plasmid and plasmids encoding one or both anthracis toxins (px01 and/or px02) can be detected by using antibiotic resistance primer sets and toxin

gene primer sets. If the *B. anthracis* is positive for tetracycline resistance, then a different antibiotic, for example quinalone, is used.

Mass spectrometry (MS)-based detection of PCR products provides all of these features with additional advantages. MS is intrinsically a parallel detection scheme without

5 the need for radioactive or fluorescent labels, since every amplification product with a unique base composition is identified by its molecular mass. The current state of the art in mass spectrometry is such that less than femtomole quantities of material can be readily analyzed to afford information about the molecular contents of the sample. An accurate assessment of the molecular mass of the material can be quickly obtained, irrespective of whether the

10 molecular weight of the sample is several hundred, or in excess of one hundred thousand atomic mass units (amu) or Daltons. Intact molecular ions can be generated from amplification products using one of a variety of ionization techniques to convert the sample to gas phase. These ionization methods include, but are not limited to, electrospray ionization (ES), matrix-assisted laser desorption ionization (MALDI) and fast atom bombardment

15 (FAB). For example, MALDI of nucleic acids, along with examples of matrices for use in MALDI of nucleic acids, are described in WO 98/54751 (Genetrace, Inc.).

In some embodiments, large DNAs and RNAs, or large amplification products therefrom, can be digested with restriction endonucleases prior to ionization. Thus, for example, an amplification product that was 10 kDa could be digested with a series of

20 restriction endonucleases to produce a panel of, for example, 100 Da fragments. Restriction endonucleases and their sites of action are well known to the skilled artisan. In this manner, mass spectrometry can be performed for the purposes of restriction mapping.

Upon ionization, several peaks are observed from one sample due to the formation of ions with different charges. Averaging the multiple readings of molecular mass obtained

25 from a single mass spectrum affords an estimate of molecular mass of the bioagent. Electrospray ionization mass spectrometry (ESI-MS) is particularly useful for very high molecular weight polymers such as proteins and nucleic acids having molecular weights greater than 10 kDa, since it yields a distribution of multiply-charged molecules of the sample without causing a significant amount of fragmentation.

30 The mass detectors used in the methods of the present invention include, but are not limited to, Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), ion trap, quadrupole, magnetic sector, time of flight (TOF), Q-TOF, and triple quadrupole.

In general, the mass spectrometric techniques which can be used in the present invention include, but are not limited to, tandem mass spectrometry, infrared multiphoton dissociation and pyrolytic gas chromatography mass spectrometry (PGC-MS). In one embodiment of the invention, the bioagent detection system operates continually in bioagent 5 detection mode using pyrolytic GC-MS without PCR for rapid detection of increases in biomass (for example, increases in fecal contamination of drinking water or of germ warfare agents). To achieve minimal latency, a continuous sample stream flows directly into the PGC-MS combustion chamber. When an increase in biomass is detected, a PCR process is automatically initiated. Bioagent presence produces elevated levels of large molecular 10 fragments from 100-7,000 Da which are observed in the PGC-MS spectrum. The observed mass spectrum is compared to a threshold level and when levels of biomass are determined to exceed a predetermined threshold, the bioagent classification process described hereinabove (combining PCR and MS, preferably FT-ICR MS) is initiated. Optionally, alarms or other processes (halting ventilation flow, physical isolation) are also initiated by this detected 15 biomass level.

The accurate measurement of molecular mass for large DNAs is limited by the adduction of cations from the PCR reaction to each strand, resolution of the isotopic peaks from natural abundance  $^{13}\text{C}$  and  $^{15}\text{N}$  isotopes, and assignment of the charge state for any ion. The cations are removed by in-line dialysis using a flow-through chip that brings the solution 20 containing the PCR products into contact with a solution containing ammonium acetate in the presence of an electric field gradient orthogonal to the flow. The latter two problems are addressed by operating with a resolving power of  $>100,000$  and by incorporating isotopically depleted nucleotide triphosphates into the DNA. The resolving power of the instrument is also a consideration. At a resolving power of 10,000, the modeled signal from the  $[\text{M}-$  25  $^{14}\text{H}+]^{14-}$  charge state of an 84mer PCR product is poorly characterized and assignment of the charge state or exact mass is impossible. At a resolving power of 33,000, the peaks from the individual isotopic components are visible. At a resolving power of 100,000, the isotopic peaks are resolved to the baseline and assignment of the charge state for the ion is straightforward. The  $^{13}\text{C}, ^{15}\text{N}$ -depleted triphosphates are obtained, for example, by growing 30 microorganisms on depleted media and harvesting the nucleotides (Batey et al., *Nucl. Acids Res.* **20**:4515-4523, 1992).

While mass measurements of intact nucleic acid regions are believed to be adequate to determine most bioagents, tandem mass spectrometry (MS<sup>n</sup>) techniques may provide more

definitive information pertaining to molecular identity or sequence. Tandem MS involves the coupled use of two or more stages of mass analysis where both the separation and detection steps are based on mass spectrometry. The first stage is used to select an ion or component of a sample from which further structural information is to be obtained. The selected ion is then 5 fragmented using, e.g., blackbody irradiation, infrared multiphoton dissociation, or collisional activation. For example, ions generated by electrospray ionization (ESI) can be fragmented using IR multiphoton dissociation. This activation leads to dissociation of glycosidic bonds and the phosphate backbone, producing two series of fragment ions, called the *w*-series (having an intact 3' terminus and a 5' phosphate following internal cleavage) and 10 the *a*-Base series(having an intact 5' terminus and a 3' furan).

The second stage of mass analysis is then used to detect and measure the mass of these resulting fragments of product ions. Such ion selection followed by fragmentation routines can be performed multiple times so as to essentially completely dissect the molecular sequence of a sample.

15 If there are two or more targets of similar base composition or mass, or if a single amplification reaction results in a product which has the same mass as two or more bioagent reference standards, they can be distinguished by using mass-modifying "tags." In this embodiment of the invention, a nucleotide analog or "tag" is incorporated during amplification (e.g., a 5-(trifluoromethyl) deoxythymidine triphosphate) which has a different 20 molecular weight than the unmodified base so as to improve distinction of masses. Such tags are described in, for example, PCT WO97/33000. This further limits the number of possible base compositions consistent with any mass. For example, 5-(trifluoromethyl)deoxythymidine triphosphate can be used in place of dTTP in a separate nucleic acid amplification reaction. Measurement of the mass shift between a conventional 25 amplification product and the tagged product is used to quantitate the number of thymidine nucleotides in each of the single strands. Because the strands are complementary, the number of adenine nucleotides in each strand is also determined.

In another amplification reaction, the number of G and C residues in each strand is determined using, for example, the cytidine analog 5-methylcytosine (5-meC) or propyne C. 30 The combination of the A/T reaction and G/C reaction, followed by molecular weight determination, provides a unique base composition. This method is summarized in Figure 4 and Table 1.

Table 1

Mass tag	Double strand sequence	Single strand Sequence	Total Δmass this strand	Base info this strand	Base info other strand	Total base comp. Top strand	Total base comp. Bottom strand
$T^* \Delta \text{mass}$ $(T^*-T) = x$	$T^* \text{ACGT}^* \text{ACGT}^*$ $\text{AT}^* \text{GCAT}^* \text{GCA}$	$T^* \text{ACGT}^* \text{ACGT}^*$	3x	3T	3A	3T	3A
		$\text{AT}^* \text{GCAT}^* \text{GCA}$	2x	2T	2A	2A	2T
$C^* \Delta \text{mass}$ $(C^*-C) = y$	$\text{TAC}^* \text{GTAC}^* \text{GT}$ $\text{ATGC}^* \text{ATGC}^* \text{A}$	$\text{TAC}^* \text{GTAC}^* \text{GT}$	2x	2C	2G	2C	2G
		$\text{ATGC}^* \text{ATGC}^* \text{A}$	2x	2C	2G	2G	2C

5 The mass tag phosphorothioate A ( $A^*$ ) was used to distinguish a *Bacillus anthracis* cluster. The *B. anthracis* ( $A_{14}G_9C_{14}T_9$ ) had an average MW of 14072.26, and the *B. anthracis* ( $A_1A^*_{13}G_9C_{14}T_9$ ) had an average molecular weight of 14281.11 and the phosphorothioate A had an average molecular weight of +16.06 as determined by ESI-TOF MS. The deconvoluted spectra are shown in Figure 5.

10 In another example, assume the measured molecular masses of each strand are 30,000.115Da and 31,000.115 Da respectively, and the measured number of dT and dA residues are (30,28) and (28,30). If the molecular mass is accurate to 100 ppm, there are 7 possible combinations of dG+dC possible for each strand. However, if the measured molecular mass is accurate to 10 ppm, there are only 2 combinations of dG+dC, and at 1 ppm accuracy there is only one possible base composition for each strand.

15 Signals from the mass spectrometer may be input to a maximum-likelihood detection and classification algorithm such as is widely used in radar signal processing. The detection processing uses matched filtering of BCS observed in mass-basecount space and allows for detection and subtraction of signatures from known, harmless organisms, and for detection of unknown bioagent threats. Comparison of newly observed bioagents to known bioagents is also possible, for estimation of threat level, by comparing their BCS to those of known

organisms and to known forms of pathogenicity enhancement, such as insertion of antibiotic resistance genes or toxin genes.

Processing may end with a Bayesian classifier using log likelihood ratios developed from the observed signals and average background levels. The program emphasizes 5 performance predictions culminating in probability-of-detection versus probability-of-false-alarm plots for conditions involving complex backgrounds of naturally occurring organisms and environmental contaminants. Matched filters consist of a priori expectations of signal values given the set of primers used for each of the bioagents. A genomic sequence database (e.g. GenBank) is used to define the mass basecount matched filters. The database contains 10 known threat agents and benign background organisms. The latter is used to estimate and subtract the signature produced by the background organisms. A maximum likelihood detection of known background organisms is implemented using matched filters and a running-sum estimate of the noise covariance. Background signal strengths are estimated and used along with the matched filters to form signatures which are then subtracted. the 15 maximum likelihood process is applied to this “cleaned up” data in a similar manner employing matched filters for the organisms and a running-sum estimate of the noise-covariance for the cleaned up data.

In one embodiment, a strategy to “triangulate” each organism by measuring signals from multiple core genes is used to reduce false negative and false positive signals, and 20 enable reconstruction of the origin or hybrid or otherwise engineered bioagents. After identification of multiple core genes, alignments are created from nucleic acid sequence databases. The alignments are then analyzed for regions of conservation and variation, and potential primer binding sites flanking variable regions are identified. Next, amplification target regions for signature analysis are selected which distinguishes organisms based on 25 specific genomic differences (i.e., base composition). For example, detection of signatures for the three part toxin genes typical of *B. anthracis* (Bowen, J.E. and C. P. Quinn, *J. Appl. Microbiol.* 1999, 87, 270-278) in the absence of the expected signatures from the *B. anthracis* genome would suggest a genetic engineering event.

The present method can also be used to detect single nucleotide polymorphisms 30 (SNPs), or multiple nucleotide polymorphisms, rapidly and accurately. A SNP is defined as a single base pair site in the genome that is different from one individual to another. The difference can be expressed either as a deletion, an insertion or a substitution, and is

frequently linked to a disease state. Because they occur every 100-1000 base pairs, SNPs are the most frequently bound type of genetic marker in the human genome.

For example, sickle cell anemia results from an A-T transition, which encodes a valine rather than a glutamic acid residue. Oligonucleotide primers may be designed such 5 that they bind to sequences that flank a SNP site, followed by nucleotide amplification and mass determination of the amplified product. Because the molecular masses of the resulting product from an individual who does not have sickle cell anemia is different from that of the product from an individual who has the disease, the method can be used to distinguish the two individuals. Thus, the method can be used to detect any known SNP in an individual and 10 thus diagnose or determine increased susceptibility to a disease or condition.

In one embodiment, blood is drawn from an individual and peripheral blood mononuclear cells (PBMC) are isolated and simultaneously tested, preferably in a high-throughput screening method, for one or more SNPs using appropriate primers based on the known sequences which flank the SNP region. The National Center for Biotechnology 15 Information maintains a publicly available database of SNPs ([www.ncbi.nlm.nih.gov/SNP/](http://www.ncbi.nlm.nih.gov/SNP/)).

The method of the present invention can also be used for blood typing. The gene encoding A, B or O blood type can differ by four single nucleotide polymorphisms. If the gene contains the sequence CGTGGTGACCCCTT (SEQ ID NO:5), antigen A results. If the gene contains the sequence CGTCGTCACCGCTA (SEQ ID NO:6) antigen B results. If the 20 gene contains the sequence CGTGGT-ACCCCTT (SEQ ID NO:7), blood group O results ("—" indicates a deletion). These sequences can be distinguished by designing a single primer pair that flanks these regions, followed by amplification and mass determination.

In other embodiments of the invention, all of the aforesaid methods can be used to determine or confirm the absence of a bioagent in a sample. For example, when the 25 molecular mass or base composition is compared to one or more molecular masses or base compositions obtained from a plurality of known organisms and no match is obtained, the absence of a particular bioagent is determined or confirmed. In these methods of determining the absence of a bioagent, a positive control can be used to confirm the integrity of the system. Such positive controls include, but are not limited to addition of a known bioagent to 30 the sample and confirming the presence of the known bioagent in the sample by obtaining a match from the comparison step. Alternately, residual primer signal can be detected, thus indicating that the system integrity is intact.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

## 5 EXAMPLES

### **Example 1: Nucleic acid isolation and PCR**

In one embodiment, nucleic acid is isolated from the organisms and amplified by PCR using standard methods prior to BCS determination by mass spectrometry. Nucleic acid is isolated, for example, by detergent lysis of bacterial cells, centrifugation and ethanol precipitation. Nucleic acid isolation methods are described in, for example, *Current Protocols in Molecular Biology* (Ausubel et al.) and *Molecular Cloning: A Laboratory Manual* (Sambrook et al.). The nucleic acid is then amplified using standard methodology, such as PCR, with primers which bind to conserved regions of the nucleic acid which contain an intervening variable sequence as described below.

15

### **Example 2: Mass spectrometry**

**FTICR Instrumentation:** The FTICR instrument is based on a 7 tesla actively shielded superconducting magnet and modified Bruker Daltonics Apex II 70e ion optics and vacuum chamber. The spectrometer is interfaced to a LEAP PAL autosampler and a custom 20 fluidics control system for high throughput screening applications. Samples are analyzed directly from 96-well or 384-well microtiter plates at a rate of about 1 sample/minute. The Bruker data-acquisition platform is supplemented with a lab-built ancillary NT datastation which controls the autosampler and contains an arbitrary waveform generator capable of generating complex rf-excite waveforms (frequency sweeps, filtered noise, stored waveform 25 inverse Fourier transform (SWIFT), etc.) for sophisticated tandem MS experiments. For oligonucleotides in the 20-30-mer regime typical performance characteristics include mass resolving power in excess of 100,000 (FWHM), low ppm mass measurement errors, and an operable *m/z* range between 50 and 5000 *m/z*.

**Modified ESI Source:** In sample-limited analyses, analyte solutions are delivered at 30 150 nL/minute to a 30 mm i.d. fused-silica ESI emitter mounted on a 3-D micromanipulator. The ESI ion optics consists of a heated metal capillary, an rf-only hexapole, a skimmer cone, and an auxiliary gate electrode. The 6.2 cm rf-only hexapole is comprised of 1 mm diameter rods and is operated at a voltage of 380 Vpp at a frequency of 5 MHz. A lab-built electro-

mechanical shutter can be employed to prevent the electrospray plume from entering the inlet capillary unless triggered to the “open” position via a TTL pulse from the data station. When in the “closed” position, a stable electrospray plume is maintained between the ESI emitter and the face of the shutter. The back face of the shutter arm contains an elastomeric seal that  
5 can be positioned to form a vacuum seal with the inlet capillary. When the seal is removed, a 1 mm gap between the shutter blade and the capillary inlet allows constant pressure in the external ion reservoir regardless of whether the shutter is in the open or closed position. When the shutter is triggered, a “time slice” of ions is allowed to enter the inlet capillary and is subsequently accumulated in the external ion reservoir. The rapid response time of the ion  
10 shutter (< 25 ms) provides reproducible, user defined intervals during which ions can be injected into and accumulated in the external ion reservoir.

**Apparatus for Infrared Multiphoton Dissociation** A 25 watt CW CO<sub>2</sub> laser operating at 10.6  $\mu$ m has been interfaced to the spectrometer to enable infrared multiphoton dissociation (IRMPD) for oligonucleotide sequencing and other tandem MS applications. An  
15 aluminum optical bench is positioned approximately 1.5 m from the actively shielded superconducting magnet such that the laser beam is aligned with the central axis of the magnet. Using standard IR-compatible mirrors and kinematic mirror mounts, the unfocused 3 mm laser beam is aligned to traverse directly through the 3.5 mm holes in the trapping electrodes of the FTICR trapped ion cell and longitudinally traverse the hexapole region of  
20 the external ion guide finally impinging on the skimmer cone. This scheme allows IRMPD to be conducted in an *m/z* selective manner in the trapped ion cell (e.g. following a SWIFT isolation of the species of interest), or in a broadband mode in the high pressure region of the external ion reservoir where collisions with neutral molecules stabilize IRMPD-generated metastable fragment ions resulting in increased fragment ion yield and sequence coverage.

25

### **Example 3: Identification of Bioagents**

Table 1 shows a small cross section of a database of calculated molecular masses for over 9 primer sets and approximately 30 organisms. The primer sets were derived from rRNA alignment. Examples of regions from rRNA consensus alignments are shown in  
30 Figures 1A-1C. Lines with arrows are examples of regions to which intelligent primer pairs for PCR are designed. The primer pairs are >95% conserved in the bacterial sequence database (currently over 10,000 organisms). The intervening regions are variable in length and/or composition, thus providing the base composition “signature” (BCS) for each

organism. Primer pairs were chosen so the total length of the amplified region is less than about 80-90 nucleotides. The label for each primer pair represents the starting and ending base number of the amplified region on the consensus diagram.

Included in the short bacterial database cross-section in Table 1 are many well known 5 pathogens/biowarfare agents (shown in bold/red typeface) such as *Bacillus anthracis* or *Yersinia pestis* as well as some of the bacterial organisms found commonly in the natural environment such as *Streptomyces*. Even closely related organisms can be distinguished from each other by the appropriate choice of primers. For instance, two low G+C organisms, *Bacillus anthracis* and *Staph aureus*, can be distinguished from each other by using the 10 primer pair defined by 16S\_1337 or 23S\_855 (M of 4 Da).

Table 2

Cross Section Of A Database Of Calculated Molecular Masses<sup>1</sup>

Primer Regions →	16S_971	16S_1100	16S_1337	16S_1294	16S_1228	23S_1021	23S_855	23S_193	23S_115
<b>Bug Name</b>									
<i>Acinetobacter calcoaceticus</i>	55619.1	55004	28446.7	35854.9	51295.4	30299	42654	39557.5	54999
<i>Bacillus anthracis</i>	<b>55005</b>	<b>54388</b>	<b>28448</b>	<b>35238</b>	<b>51296</b>	<b>30295</b>	<b>42651</b>	<b>39560</b>	<b>56850</b>
<i>Bacillus cereus</i>	55622.1	54387.9	28447.6	35854.9	51296.4	30295	42651	39560.5	56850.3
<i>Bordetella bronchiseptica</i>	56857.3	51300.4	28446.7	35857.9	51307.4	30299	42653	39559.5	51920.5
<i>Borrelia burgdorferi</i>	56231.2	55621.1	28440.7	35852.9	51295.4	30297	42029.9	38941.4	52524.6
<i>Brucella abortus</i>	<b>58098</b>	<b>55011</b>	<b>28448</b>	<b>35854</b>	<b>50683</b>				
<i>Campylobacter jejuni</i>	58088.5	54386.9	29061.8	35856.9	50674.3	30294	42032.9	39558.5	45732.5
<i>Chlamydia pneumoniae</i>	<b>55000</b>	<b>55007</b>	<b>29063</b>	<b>35855</b>	<b>50676</b>	<b>30295</b>	<b>42036</b>	<b>38941</b>	<b>56230</b>
<i>Clostridium botulinum</i>	<b>55006</b>	<b>53767</b>	<b>28445</b>	<b>35855</b>	<b>51291</b>	<b>30300</b>	<b>42656</b>	<b>39562</b>	<b>54999</b>
<i>Clostridium difficile</i>	56855.3	54386.9	28444.7	35853.9	51296.4	30294	41417.8	39556.5	55612.2
<i>Enterococcus faecalis</i>	55620.1	54387.9	28447.6	35858.9	51296.4	30297	42652	39559.5	56849.3
<i>Escherichia coli</i>	<b>55622</b>	<b>55009</b>	<b>28445</b>	<b>35857</b>	<b>51301</b>	<b>30301</b>	<b>42656</b>	<b>39562</b>	<b>54999</b>
<i>Francisella tularensis</i>	<b>53769</b>	<b>54385</b>	<b>28445</b>	<b>35856</b>	<b>51298</b>				
<i>Haemophilus influenzae</i>	55620.1	55006	28444.7	35855.9	51298.4	30298	42656	39560.5	55613.1
<i>Klebsiella pneumoniae</i>	55622.1	55008	28442.7	35856.9	51297.4	30300	42655	39562.5	55000
<i>Legionella pneumophila</i>	<b>55618</b>	<b>55626</b>	<b>28446</b>	<b>35857</b>	<b>51303</b>				
<i>Mycobacterium avium</i>	54390.9	55631.1	29064.8	35858.9	51915.5	30298	42656	38942.4	56241.2
<i>Mycobacterium leprae</i>	54389.9	55629.1	29064.8	35860.9	51917.5	30298	42656	39559.5	56240.2
<i>Mycobacterium tuberculosis</i>	54390.9	55629.1	29064.8	35860.9	51301.4	30299	42656	39560.5	56243.2
<i>Mycoplasma genitalium</i>	53143.7	45115.4	29061.8	35854.9	50671.3	30294	43264.1	39558.5	56842.4
<i>Mycoplasma pneumoniae</i>	53143.7	45118.4	29061.8	35854.9	50673.3	30294	43264.1	39559.5	56843.4
<i>Neisseria gonorrhoeae</i>	55627.1	54389.9	28445.7	35855.9	51302.4	30300	42649	39561.5	55000
<i>Pseudomonas aeruginosa</i>	<b>55623</b>	<b>55010</b>	<b>28443</b>	<b>35858</b>	<b>51301</b>	<b>30298</b>	<b>43272</b>	<b>39558</b>	<b>55619</b>
<i>Rickettsia prowazekii</i>	<b>58093</b>	<b>55621</b>	<b>28448</b>	<b>35853</b>	<b>50677</b>	<b>30293</b>	<b>42650</b>	<b>39559</b>	<b>53139</b>
<i>Rickettsia rickettsii</i>	<b>58094</b>	<b>55623</b>	<b>28448</b>	<b>35853</b>	<b>50679</b>	<b>30293</b>	<b>42648</b>	<b>39559</b>	<b>53755</b>
<i>Salmonella typhimurium</i>	<b>55622</b>	<b>55005</b>	<b>28445</b>	<b>35857</b>	<b>51301</b>	<b>30301</b>	<b>42658</b>		
<i>Shigella dysenteriae</i>	<b>55623</b>	<b>55009</b>	<b>28444</b>	<b>35857</b>	<b>51301</b>				
<i>Staphylococcus aureus</i>	56854.3	54386.9	28443.7	35852.9	51294.4	30298	42655	39559.5	57466.4
<i>Streptomyces</i>	54389.9	59341.6	29063.8	35858.9	51300.4			39563.5	56864.3
<i>Treponema pallidum</i>	56245.2	55631.1	28445.7	35851.9	51297.4	30299	42034.9	38939.4	57473.4
<i>Vibrio cholerae</i>	<b>55625</b>	<b>55626</b>	<b>28443</b>	<b>35857</b>	<b>52536</b>	<b>29063</b>	<b>30303</b>	<b>35241</b>	<b>50675</b>
<i>Vibrio parahaemolyticus</i>	54384.9	55626.1	28444.7	34620.7	50064.2				
<i>Yersinia pestis</i>	<b>55620</b>	<b>55626</b>	<b>28443</b>	<b>35857</b>	<b>51299</b>				

<sup>1</sup>Molecular mass distribution of PCR amplified regions for a selection of organisms (rows) 15 across various primer pairs (columns). Pathogens are shown in bold. Empty cells indicate presently incomplete or missing data.

Figure 6 shows the use of ESI-FT-ICR MS for measurement of exact mass. The spectra from 46mer PCR products originating at position 1337 of the 16S rRNA from *S.*

*aureus* (upper) and *B. anthracis* (lower) are shown. These data are from the region of the spectrum containing signals from the  $[M-8H^+]^{8-}$  charge states of the respective 5'-3' strands. The two strands differ by two (AT $\rightarrow$ CG) substitutions, and have measured masses of 14206.396 and  $14208.373 \pm 0.010$  Da, respectively. The possible base compositions derived 5 from the masses of the forward and reverse strands for the *B. anthracis* products are listed in Table 3.

**Table 3**  
**Possible base composition for *B. anthracis* products**

Calc. Mass	Error	Base Comp.
14208.2935	0.079520	A1 G17 C10 T18
14208.3160	0.056980	A1 G20 C15 T10
14208.3386	0.034440	A1 G23 C20 T2
14208.3074	0.065560	A6 G11 C3 T26
14208.3300	0.043020	A6 G14 C8 T18
14208.3525	0.020480	A6 G17 C13 T10
14208.3751	0.002060	A6 G20 C18 T2
14208.3439	0.029060	A11 G8 C1 T26
14208.3665	0.006520	A11 G11 C6 T18
<b>14208.3890</b>	<b>0.016020</b>	<b>A11 G14 C11 T10</b>
14208.4116	0.038560	A11 G17 C16 T2
14208.4030	0.029980	A16 G8 C4 T18
14208.4255	0.052520	A16 G11 C9 T10
14208.4481	0.075060	A16 G14 C14 T2
14208.4395	0.066480	A21 G5 C2 T18
14208.4620	0.089020	A21 G8 C7 T10
14079.2624	0.080600	A0 G14 C13 T19
14079.2849	0.058060	A0 G17 C18 T11
14079.3075	0.035520	A0 G20 C23 T3
14079.2538	0.089180	A5 G5 C1 T35
14079.2764	0.066640	A5 G8 C6 T27
14079.2989	0.044100	A5 G11 C11 T19
14079.3214	0.021560	A5 G14 C16 T11

14079.3440	0.000980	A5 G17 C21 T3
14079.3129	0.030140	A10 G5 C4 T27
14079.3354	0.007600	A10 G8 C9 T19
<b>14079.3579</b>	<b>0.014940</b>	<b>A10 G11 C14 T11</b>
14079.3805	0.037480	A10 G14 C19 T3
14079.3494	0.006360	A15 G2 C2 T27
14079.3719	0.028900	A15 G5 C7 T19
14079.3944	0.051440	A15 G8 C12 T11
14079.4170	0.073980	A15 G11 C17 T3
14079.4084	0.065400	A20 G2 C5 T19
14079.4309	0.087940	A20 G5 C10 T13

Among the 16 compositions for the forward strand and the 18 compositions for the reverse strand that were calculated, only one pair (shown in **bold**) are complementary, corresponding to the actual base compositions of the *B. anthracis* PCR products.

5

#### Example 4: BCS of Region from *Bacillus anthracis* and *Bacillus cereus*

A conserved *Bacillus* region from *B. anthracis* (A<sub>14</sub>G<sub>9</sub>C<sub>14</sub>T<sub>9</sub>) and *B. cereus* (A<sub>15</sub>G<sub>9</sub>C<sub>13</sub>T<sub>9</sub>) having a C to A base change was synthesized and subjected to ESI-TOF MS. The results are shown in Figure 7 in which the two regions are clearly distinguished using the method of the present invention (MW=14072.26 vs. 14096.29).

#### Example 5: Identification of Additional Bioagents

In other examples of the present invention, the pathogen *Vibrio cholera* can be distinguished from *Vibrio parahemolyticus* with  $\Delta M > 600$  Da using one of three 16S primer sets shown in Table 2 (16S\_971, 16S\_1228 or 16S\_1294) as shown in Table 4. The two mycoplasma species in the list (*M. genitalium* and *M. pneumoniae*) can also be distinguished from each other, as can the three mycobacteriae. While the direct mass measurements of amplified products can identify and distinguish a large number of organisms, measurement of the base composition signature provides dramatically enhanced resolving power for closely related organisms. In cases such as *Bacillus anthracis* and *Bacillus cereus* that are virtually indistinguishable from each other based solely on mass differences, compositional analysis or

fragmentation patterns are used to resolve the differences. The single base difference between the two organisms yields different fragmentation patterns, and despite the presence of the ambiguous/unidentified base N at position 20 in *B. anthracis*, the two organisms can be identified.

5 Tables 4a-b show examples of primer pairs from Table 1 which distinguish pathogens from background.

**Table 4a**

Organism name	23S_855	16S_1337	23S_1021
<i>Bacillus anthracis</i>	42650.98	28447.65	30294.98
<i>Staphylococcus aureus</i>	42654.97	28443.67	30297.96

**Table 4b**

Organism name	16S_971	16S_1294	16S_1228
<i>Vibrio cholerae</i>	55625.09	35856.87	52535.59
<i>Vibrio parahaemolyticus</i>	54384.91	34620.67	50064.19

10

Table 4 shows the expected molecular weight and base composition of region 16S\_1100-1188 in *Mycobacterium avium* and *Streptomyces sp.*

**Table 5**

Region	Organism name	Length	Molecular weight	Base comp.
16S_1100-1188	<i>Mycobacterium avium</i>	82	25624.1728	A <sub>16</sub> G <sub>32</sub> C <sub>18</sub> T <sub>16</sub>
16S_1100-1188	<i>Streptomyces sp.</i>	96	29904.871	A <sub>17</sub> G <sub>38</sub> C <sub>27</sub> T <sub>14</sub>

15

Table 5 shows base composition (single strand) results for 16S\_1100-1188 primer amplification reactions different species of bacteria. Species which are repeated in the table (e.g., *Clostridium botulinum*) are different strains which have different base compositions in the 16S\_1100-1188 region.

20

**Table 6**

Organism name	Base comp.	Organism name	Base comp.
<i>Mycobacterium avium</i>	A <sub>16</sub> G <sub>32</sub> C <sub>18</sub> T <sub>16</sub>	<i>Vibrio cholerae</i>	A <sub>23</sub> G <sub>30</sub> C <sub>21</sub> T <sub>16</sub>

Streptomyces sp.	A <sub>17</sub> G <sub>38</sub> C <sub>27</sub> T <sub>14</sub>	<b>Aeromonas hydrophila</b>	A <sub>23</sub> G <sub>31</sub> C <sub>21</sub> T <sub>15</sub>
Ureaplasma urealyticum	A <sub>18</sub> G <sub>30</sub> C <sub>17</sub> T <sub>17</sub>	<b>Aeromonas salmonicida</b>	A <sub>23</sub> G <sub>31</sub> C <sub>21</sub> T <sub>15</sub>
Streptomyces sp.	A <sub>19</sub> G <sub>36</sub> C <sub>24</sub> T <sub>18</sub>	Mycoplasma genitalium	A <sub>24</sub> G <sub>19</sub> C <sub>12</sub> T <sub>18</sub>
Mycobacterium leprae	A <sub>20</sub> G <sub>32</sub> C <sub>22</sub> T <sub>16</sub>	Clostridium botulinum	A <sub>24</sub> G <sub>25</sub> C <sub>18</sub> T <sub>20</sub>
<b>M. tuberculosis</b>	<b>A<sub>20</sub>G<sub>33</sub>C<sub>21</sub>T<sub>16</sub></b>	<b>Bordetella bronchiseptica</b>	A <sub>24</sub> G <sub>26</sub> C <sub>19</sub> T <sub>14</sub>
<b>Nocardia asteroides</b>	<b>A<sub>20</sub>G<sub>33</sub>C<sub>21</sub>T<sub>16</sub></b>	Francisella tularensis	A <sub>24</sub> G <sub>26</sub> C <sub>19</sub> T <sub>19</sub>
Fusobacterium necroforum	A <sub>21</sub> G <sub>26</sub> C <sub>22</sub> T <sub>18</sub>	<b>Bacillus anthracis</b>	A <sub>24</sub> G <sub>26</sub> C <sub>20</sub> T <sub>18</sub>
Listeria monocytogenes	A <sub>21</sub> G <sub>27</sub> C <sub>19</sub> T <sub>19</sub>	<b>Campylobacter jejuni</b>	A <sub>24</sub> G <sub>26</sub> C <sub>20</sub> T <sub>18</sub>
Clostridium botulinum	A <sub>21</sub> G <sub>27</sub> C <sub>19</sub> T <sub>21</sub>	<b>Staphylococcus aureus</b>	A <sub>24</sub> G <sub>26</sub> C <sub>20</sub> T <sub>18</sub>
Neisseria gonorrhoeae	A <sub>21</sub> G <sub>28</sub> C <sub>21</sub> T <sub>18</sub>	Helicobacter pylori	A <sub>24</sub> G <sub>26</sub> C <sub>20</sub> T <sub>19</sub>
Bartonella quintana	A <sub>21</sub> G <sub>30</sub> C <sub>22</sub> T <sub>16</sub>	Helicobacter pylori	A <sub>24</sub> G <sub>26</sub> C <sub>21</sub> T <sub>18</sub>
Enterococcus faecalis	A <sub>22</sub> G <sub>27</sub> C <sub>20</sub> T <sub>19</sub>	Moraxella catarrhalis	A <sub>24</sub> G <sub>26</sub> C <sub>23</sub> T <sub>16</sub>
Bacillus megaterium	A <sub>22</sub> G <sub>28</sub> C <sub>20</sub> T <sub>18</sub>	Haemophilus influenzae Rd	A <sub>24</sub> G <sub>28</sub> C <sub>20</sub> T <sub>17</sub>
Bacillus subtilis	A <sub>22</sub> G <sub>28</sub> C <sub>21</sub> T <sub>17</sub>	<b>Chlamydia trachomatis</b>	A <sub>24</sub> G <sub>28</sub> C <sub>21</sub> T <sub>16</sub>
Pseudomonas aeruginosa	A <sub>22</sub> G <sub>29</sub> C <sub>23</sub> T <sub>15</sub>	<b>Chlamydophila pneumoniae</b>	A <sub>24</sub> G <sub>28</sub> C <sub>21</sub> T <sub>16</sub>
Legionella pneumophila	A <sub>22</sub> G <sub>32</sub> C <sub>20</sub> T <sub>16</sub>	<b>C. pneumonia AR39</b>	A <sub>24</sub> G <sub>28</sub> C <sub>21</sub> T <sub>16</sub>
Mycoplasma pneumoniae	A <sub>23</sub> G <sub>20</sub> C <sub>14</sub> T <sub>16</sub>	Pseudomonas putida	A <sub>24</sub> G <sub>29</sub> C <sub>21</sub> T <sub>16</sub>
Clostridium botulinum	A <sub>23</sub> G <sub>26</sub> C <sub>20</sub> T <sub>19</sub>	<b>Proteus vulgaris</b>	A <sub>24</sub> G <sub>30</sub> C <sub>21</sub> T <sub>15</sub>
Enterococcus faecium	A <sub>23</sub> G <sub>26</sub> C <sub>21</sub> T <sub>18</sub>	<b>Yersinia pestis</b>	A <sub>24</sub> G <sub>30</sub> C <sub>21</sub> T <sub>15</sub>
Acinetobacter calcoaceti	A <sub>23</sub> G <sub>26</sub> C <sub>21</sub> T <sub>19</sub>	<b>Yersinia pseudotuberculosis</b>	A <sub>24</sub> G <sub>30</sub> C <sub>21</sub> T <sub>15</sub>
Leptospira borgpeterseni	A <sub>23</sub> G <sub>26</sub> C <sub>24</sub> T <sub>15</sub>	Clostridium botulinum	A <sub>25</sub> G <sub>24</sub> C <sub>18</sub> T <sub>21</sub>
Leptospira interrogans	A <sub>23</sub> G <sub>26</sub> C <sub>24</sub> T <sub>15</sub>	Clostridium tetani	A <sub>25</sub> G <sub>25</sub> C <sub>18</sub> T <sub>20</sub>
Clostridium perfringens	A <sub>23</sub> G <sub>27</sub> C <sub>19</sub> T <sub>19</sub>	Francisella tularensis	A <sub>25</sub> G <sub>25</sub> C <sub>19</sub> T <sub>19</sub>
Bacillus anthracis	A <sub>23</sub> G <sub>27</sub> C <sub>20</sub> T <sub>18</sub>	Acinetobacter calcoacetic	A <sub>25</sub> G <sub>26</sub> C <sub>20</sub> T <sub>19</sub>
Bacillus cereus	A <sub>23</sub> G <sub>27</sub> C <sub>20</sub> T <sub>18</sub>	Bacteriodes fragilis	A <sub>25</sub> G <sub>27</sub> C <sub>16</sub> T <sub>22</sub>
Bacillus thuringiensis	A <sub>23</sub> G <sub>27</sub> C <sub>20</sub> T <sub>18</sub>	Chlamydophila psittaci	A <sub>25</sub> G <sub>27</sub> C <sub>21</sub> T <sub>16</sub>
<i>Aeromonas hydrophila</i>	A <sub>23</sub> G <sub>29</sub> C <sub>21</sub> T <sub>16</sub>	Borrelia burgdorferi	A <sub>25</sub> G <sub>29</sub> C <sub>17</sub> T <sub>19</sub>
<i>Escherichia coli</i>	A <sub>23</sub> G <sub>29</sub> C <sub>21</sub> T <sub>16</sub>	Streptobacillus monilifor	A <sub>26</sub> G <sub>26</sub> C <sub>20</sub> T <sub>16</sub>
Pseudomonas putida	A <sub>23</sub> G <sub>29</sub> C <sub>21</sub> T <sub>17</sub>	Rickettsia prowazekii	A <sub>26</sub> G <sub>28</sub> C <sub>18</sub> T <sub>18</sub>
<i>Escherichia coli</i>	A <sub>23</sub> G <sub>29</sub> C <sub>22</sub> T <sub>15</sub>	Rickettsia rickettsii	A <sub>26</sub> G <sub>28</sub> C <sub>20</sub> T <sub>16</sub>

Shigella dysenteriae	<b>A<sub>23</sub>G<sub>29</sub>C<sub>22</sub>T<sub>15</sub></b>	Mycoplasma mycoides	A <sub>28</sub> G <sub>23</sub> C <sub>16</sub> T <sub>20</sub>
----------------------	-----------------------------------------------------------------	---------------------	-----------------------------------------------------------------

The same organism having different base compositions are different strains. Groups of organisms which are highlighted or in italics have the same base compositions in the amplified region. Some of these organisms can be distinguished using multiple primers. For 5 example, *Bacillus anthracis* can be distinguished from *Bacillus cereus* and *Bacillus thuringiensis* using the primer 16S\_971-1062 (Table 6). Other primer pairs which produce unique base composition signatures are shown in Table 6 (bold). Clusters containing very similar threat and ubiquitous non-threat organisms (e.g. *anthracis* cluster) are distinguished at high resolution with focused sets of primer pairs. The known biowarfare agents in Table 6 10 are *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis* and *Rickettsia prowazekii*.

Table 7

Organism	16S_971-1062	16S_1228-1310	16S_1100-1188
<i>Aeromonas hydrophila</i>	A <sub>21</sub> G <sub>29</sub> C <sub>22</sub> T <sub>20</sub>	A <sub>22</sub> G <sub>27</sub> C <sub>21</sub> T <sub>13</sub>	A <sub>23</sub> G <sub>31</sub> C <sub>21</sub> T <sub>15</sub>
<i>Aeromonas salmonicida</i>	A <sub>21</sub> G <sub>29</sub> C <sub>22</sub> T <sub>20</sub>	A <sub>22</sub> G <sub>27</sub> C <sub>21</sub> T <sub>13</sub>	A <sub>23</sub> G <sub>31</sub> C <sub>21</sub> T <sub>15</sub>
<i>Bacillus anthracis</i>	<b>A<sub>21</sub>G<sub>27</sub>C<sub>22</sub>T<sub>22</sub></b>	A <sub>24</sub> G <sub>22</sub> C <sub>19</sub> T <sub>18</sub>	A <sub>23</sub> G <sub>27</sub> C <sub>20</sub> T <sub>18</sub>
<i>Bacillus cereus</i>	A <sub>22</sub> G <sub>27</sub> C <sub>21</sub> T <sub>22</sub>	A <sub>24</sub> G <sub>22</sub> C <sub>19</sub> T <sub>18</sub>	A <sub>23</sub> G <sub>27</sub> C <sub>20</sub> T <sub>18</sub>
<i>Bacillus thuringiensis</i>	A <sub>22</sub> G <sub>27</sub> C <sub>21</sub> T <sub>22</sub>	A <sub>24</sub> G <sub>22</sub> C <sub>19</sub> T <sub>18</sub>	A <sub>23</sub> G <sub>27</sub> C <sub>20</sub> T <sub>18</sub>
<i>Chlamydia trachomatis</i>	A <sub>22</sub> G <sub>26</sub> C <sub>20</sub> T <sub>23</sub>	<b>A<sub>24</sub>G<sub>23</sub>C<sub>19</sub>T<sub>16</sub></b>	A <sub>24</sub> G <sub>28</sub> C <sub>21</sub> T <sub>16</sub>
<i>Chlamydia pneumoniae AR39</i>	A <sub>26</sub> G <sub>23</sub> C <sub>20</sub> T <sub>22</sub>	A <sub>26</sub> G <sub>22</sub> C <sub>16</sub> T <sub>18</sub>	A <sub>24</sub> G <sub>28</sub> C <sub>21</sub> T <sub>16</sub>
<i>Leptospira borgpetersenii</i>	A <sub>22</sub> G <sub>26</sub> C <sub>20</sub> T <sub>21</sub>	A <sub>22</sub> G <sub>25</sub> C <sub>21</sub> T <sub>15</sub>	A <sub>23</sub> G <sub>26</sub> C <sub>24</sub> T <sub>15</sub>
<i>Leptospira interrogans</i>	A <sub>22</sub> G <sub>26</sub> C <sub>20</sub> T <sub>21</sub>	A <sub>22</sub> G <sub>25</sub> C <sub>21</sub> T <sub>15</sub>	A <sub>23</sub> G <sub>26</sub> C <sub>24</sub> T <sub>15</sub>
<i>Mycoplasma genitalium</i>	A <sub>28</sub> G <sub>23</sub> C <sub>15</sub> T <sub>22</sub>	<b>A<sub>30</sub>G<sub>18</sub>C<sub>15</sub>T<sub>19</sub></b>	<b>A<sub>24</sub>G<sub>19</sub>C<sub>12</sub>T<sub>18</sub></b>
<i>Mycoplasma pneumoniae</i>	A <sub>28</sub> G <sub>23</sub> C <sub>15</sub> T <sub>22</sub>	<b>A<sub>27</sub>G<sub>19</sub>C<sub>16</sub>T<sub>20</sub></b>	<b>A<sub>23</sub>G<sub>20</sub>C<sub>14</sub>T<sub>16</sub></b>
<i>Escherichia coli</i>	<b>A<sub>22</sub>G<sub>28</sub>C<sub>20</sub>T<sub>22</sub></b>	A <sub>24</sub> G <sub>25</sub> C <sub>21</sub> T <sub>13</sub>	A <sub>23</sub> G <sub>29</sub> C <sub>22</sub> T <sub>15</sub>
<i>Shigella dysenteriae</i>	<b>A<sub>22</sub>G<sub>28</sub>C<sub>21</sub>T<sub>21</sub></b>	A <sub>24</sub> G <sub>25</sub> C <sub>21</sub> T <sub>13</sub>	A <sub>23</sub> G <sub>29</sub> C <sub>22</sub> T <sub>15</sub>
<i>Proteus vulgaris</i>	<b>A<sub>23</sub>G<sub>26</sub>C<sub>22</sub>T<sub>21</sub></b>	<b>A<sub>26</sub>G<sub>24</sub>C<sub>19</sub>T<sub>14</sub></b>	A <sub>24</sub> G <sub>30</sub> C <sub>21</sub> T <sub>15</sub>
<i>Yersinia pestis</i>	A <sub>24</sub> G <sub>25</sub> C <sub>21</sub> T <sub>22</sub>	A <sub>25</sub> G <sub>24</sub> C <sub>20</sub> T <sub>14</sub>	A <sub>24</sub> G <sub>30</sub> C <sub>21</sub> T <sub>15</sub>
<i>Yersinia pseudotuberculosis</i>	A <sub>24</sub> G <sub>25</sub> C <sub>21</sub> T <sub>22</sub>	A <sub>25</sub> G <sub>24</sub> C <sub>20</sub> T <sub>14</sub>	A <sub>24</sub> G <sub>30</sub> C <sub>21</sub> T <sub>15</sub>
<i>Francisella tularensis</i>	<b>A<sub>20</sub>G<sub>25</sub>C<sub>21</sub>T<sub>23</sub></b>	<b>A<sub>23</sub>G<sub>26</sub>C<sub>17</sub>T<sub>17</sub></b>	<b>A<sub>24</sub>G<sub>26</sub>C<sub>19</sub>T<sub>19</sub></b>
<i>Rickettsia prowazekii</i>	<b>A<sub>21</sub>G<sub>26</sub>C<sub>24</sub>T<sub>25</sub></b>	<b>A<sub>24</sub>G<sub>23</sub>C<sub>16</sub>T<sub>19</sub></b>	<b>A<sub>26</sub>G<sub>28</sub>C<sub>18</sub>T<sub>18</sub></b>
<i>Rickettsia rickettsii</i>	<b>A<sub>21</sub>G<sub>26</sub>C<sub>25</sub>T<sub>24</sub></b>	<b>A<sub>24</sub>G<sub>24</sub>C<sub>17</sub>T<sub>17</sub></b>	<b>A<sub>26</sub>G<sub>28</sub>C<sub>20</sub>T<sub>16</sub></b>

The sequence of *B. anthracis* and *B. cereus* in region 16S\_971 is shown below. Shown in bold is the single base difference between the two species which can be detected using the methods of the present invention. *B. anthracis* has an ambiguous base at position 20.

5 *B.anthracis\_16S\_971*

GC<sup>GA</sup>AGAACCUUACCAGGUNUGACAUCCUCUGACACCCUAGAGAUAGGGCU  
UCUCCUUCGGGAGCAGAGUGACAGGUGGUGCAUGGUU (SEQ ID NO: 1)

*B.cereus\_16S\_971*

GC<sup>GA</sup>AGAACCUUACCAGGUCUGACAUCCUCUGAAACCCUAGAGAUAGGGCU

10 UCUCCUUCGGGAGCAGAGUGACAGGUGGUGCAUGGUU (SEQ ID NO: 2)

**Example 6: ESI-TOF MS of sspE 56-mer Plus Calibrant**

The mass measurement accuracy that can be obtained using an internal mass standard in the ESI-MS study of PCR products is shown in Fig.8. The mass standard was a 20-mer 15 phosphorothioate oligonucleotide added to a solution containing a 56-mer PCR product from the *B. anthracis* spore coat protein sspE. The mass of the expected PCR product distinguishes *B. anthracis* from other species of Bacillus such as *B. thuringiensis* and *B. cereus*.

20 **Example 7: *B. anthracis* ESI-TOF Synthetic 16S\_1228 Duplex**

An ESI-TOF MS spectrum was obtained from an aqueous solution containing 5  $\mu$ M each of synthetic analogs of the expected forward and reverse PCR products from the nucleotide 1228 region of the *B. anthracis* 16S rRNA gene. The results (Fig. 9) show that the molecular weights of the forward and reverse strands can be accurately determined and easily 25 distinguish the two strands. The  $[M-21H^+]^{21-}$  and  $[M-20H^+]^{20-}$  charge states are shown.

**Example 8: ESI-FTICR-MS of Synthetic *B. anthracis* 16S\_1337 46 Base Pair Duplex**

An ESI-FTICR-MS spectrum was obtained from an aqueous solution containing 5  $\mu$ M each of synthetic analogs of the expected forward and reverse PCR products from the 30 nucleotide 1337 region of the *B. anthracis* 16S rRNA gene. The results (Fig. 10) show that the molecular weights of the strands can be distinguished by this method. The  $[M-16H^+]^{16-}$  through  $[M-10H^+]^{10-}$  charge states are shown. The insert highlights the resolution that can be

realized on the FTICR-MS instrument, which allows the charge state of the ion to be determined from the mass difference between peaks differing by a single <sup>13</sup>C substitution.

**Example 9: ESI-TOF MS of 56-mer Oligonucleotide from saspB Gene of *B. anthracis***

5 **with Internal Mass Standard**

ESI-TOF MS spectra were obtained on a synthetic 56-mer oligonucleotide (5  $\mu$ M) from the saspB gene of *B. anthracis* containing an internal mass standard at an ESI of 1.7  $\mu$ L/min as a function of sample consumption. The results (Fig. 11) show that the signal to noise is improved as more scans are summed, and that the standard and the product are 10 visible after only 100 scans.

**Example 10: ESI-TOF MS of an Internal Standard with Tributylammonium (TBA)-trifluoroacetate (TFA) Buffer**

An ESI-TOF-MS spectrum of a 20-mer phosphorothioate mass standard was obtained 15 following addition of 5 mM TBA-TFA buffer to the solution. This buffer strips charge from the oligonucleotide and shifts the most abundant charge state from  $[M-8H^+]$ <sup>8-</sup> to  $[M-3H^+]$ <sup>3-</sup> (Fig. 12).

Various modifications of the invention, in addition to those described herein, will be 20 apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. Each reference cited in the present application is incorporated herein by reference in its entirety.